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RESEARCH ARTICLE

# Evidence for Intraaxonal Spread of *Listeria Monocytogenes* from the Periphery to the Central Nervous System

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**Rhombencephalitis due to *Listeria monocytogenes* is characterized by progressive cranial nerve palsies and subacute inflammation in the brain stem. In this paper, we report observations made on mice infected with *L. monocytogenes*. Unilateral inoculation of bacteria into facial muscle, or peripheral parts of a cranial nerve, induced clinical and histological signs of mainly ipsilateral rhombencephalitis. Similarly, unilateral inoculation of bacteria into lower leg muscle or peripheral parts of sciatic nerve was followed by lumbar myelitis. In these animals, intraaxonal bacteria were seen in the sciatic nerve and its corresponding nerve roots ipsilateral to the bacterial application site. Development of myelitis was prevented by transsection of the sciatic nerve proximally to the hindleg inoculation site. Altogether, our results support the hypothesis that Listeria rhombencephalitis is caused by intraaxonal bacterial spread from peripheral sites to the central nervous system.**

## Introduction

Listeriosis is caused by the facultatively intracellular Gram positive bacterium *Listeria monocytogenes*. Human listeriosis includes cases of septicemia, meningitis, and meningoencephalitis. The disease occurs sporadically or in epidemics, and mainly affects neonates, pregnant, elderly, or immunosuppressed individuals (15, 24). Rhombencephalitis is a rare variant which may affect immunocompetent individuals (2). It is common in sheep and other ruminant species (25). In human cases, flu-like symptoms are followed by an abrupt onset of progressive asymmetric cranial nerve deficits — with or without more widespread brain involvement and meningeal signs. The condition is fatal if not treated with antibiotics (2, 38). Cranial nerve deficits, hemiparesis, and ataxia are common sequelae in surviving patients (2). Histopathological studies reveal subacute encephalitis with micro-abscesses in cranial nerve nuc1ei, variably in combination with meningitis and/or more widespread encephalitis (2).

The predilection of *L. monocytogenes* for brain stem areas is not understood. One explanation would be a hematogenous spread of the bacterium combined with a tropism for brain stem areas. A brain stem tropism would enable the organism to give rise to rhombencephalitis after its crossing of the blood-brain barrier. An alternative hypothesis suggests that *L. monocytogenes* spread along cranial nerves, directly from peripheral sites to the parenchyma of the brain stem (3, 7, 8, 27).

We present observations made on mice infected with *L. monocytogenes*. In this animal, bacterial inoculation into peripheral tissues leads to focal infection in the corresponding areas of the central nervous system.

## Materials and Methods

The animal experiments were approved by the Norwegian animal research committee (Forsøksdyrutvalget). Fifty-six female Hsd/ICR mice weighing 25-35 g (Harlan, UK) were anesthetized with a combination of fentanyl 0.5 mg/ml, fluanisone 2.5 mg/ml (Hypnorm, Janssen Beerse, Belgium), and midazolam 1.25 mg/ml (Dormicum, Roche, Basel, Switzerland) injected subcutaneously (6 ml/kg body weight). A local isolate of *L. monocytogenes* serotype 4 was used (Table 1). In 22 animals (groups A, C, E; table 1) $6 \times 10^5$ bacteria in 20 μl saline were injected into skeletal muscle (facial or triceps surae muscle). In group E, the sciatic nerve was interrupted at the mid-thigh level prior to bacterial inoculation into triceps surae muscle. In the other 34 animals

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| Group | Experimental Procedure | Total Number | *Neuro-Listeriosis | Dead/ Systemic Listeriosis | No Disease |
|---|---|---|---|---|---|
| **A** | Inoculation into facial muscle in the whisker region. | 7 | 4 | 1 | 2 |
| **B** | Inoculation into proximal end of cut facial nerve. | 19 | 9 | 5 | 5 |
| **C** | Inoculation into triceps surae muscle. | 10 | 6 | 4 | - |
| **D** | Inoculation into proximal end of cut sciatic nerve. | 15 | 9 | - | 6 |
| **E** | Inoculation into triceps surae muscle after sciatic nerve section in the mid-thigh level. | 5 | - | - | 5 |
| **All groups** | | 56 | 28 | 10 | 18 |

* Neurolisteriosis in groups A and B: Rhombencephalitis.
Neurolisteriosis in groups C and D: Transverse myelitis.

**Table 1.** Experimental inoculation of mice with Listeria monocytogenes; clinical outcome.

(groups B and D; table 1), $3 \times 10^6$ bacteria in 10 µl saline were applied to the proximal ending of a cut nerve (facial or sciatic nerve). In some of these experiments, the proximal nerve ending was drawn into a close fitting suction pipette prior to bacterial exposure in order to reduce spread of bacteria into perineural tissues.

Animals with weight loss, ruffled fur, and/or neurological symptoms were killed with a subcutaneous overdose of anesthetics, and perfused through the heart with 4% phosphate-buffered formaldehyde. Tissue samples from the central nervous system and peripheral nerves were embedded in paraffin, and 5 µm microscopical sections were cut and stained with haematoxylin and eosin (H & E). Parallel sections were immunostained for *L. monocytogenes* with a rabbit anti-listeria serotype 4 antiserum; dilution 1: 1280 (DifCo laboratories, USA); followed by application of an indirect biotin avidine system for detection of the primary antibody binding (Ventana Detection Kit, Ventana Medical Systems, USA).

## Results

Twenty-eight animals developed signs of neurolisteriosis. These signs occurred after 5-7 days (rhombencephalitis, groups A and B in table 1), and 7-10 days (transverse myelitis, groups C and D in table 1), respectively. Figure 1 depicts experimental rhombencephalitis with inflammation in cranial nerve nuclei. The highest rate of neurolisteriosis occurred after selective inoculation of the bacteria to proximal nerve stumps drawn into a suction pipette. This procedure also reduced the number of animals developing symptoms of systemic disease.

In addition to the animals developing signs of neurolisteriosis, 4 animals were found dead in their cage without preceding symptoms (three to seven days after inoculation) and 6 animals developed symptoms of severe disease with weight loss and ruffled fur (Table 1). Since microscopical examination revealed microabscesses with numerous bacteria in liver and spleen, the latter was probably due to systemic disease.

### Bacterial inoculation into facial muscle and facial nerve (groups A and B)

Clinical signs of brain stem infection developed five to seven days after unilateral inoculation of bacteria into facial muscle or the proximal end of cut facial nerve. Unilateral brain stem symptoms dominated in the early phase. In animals with intact facial nerve, initial symptoms were ptosis and paralysis of whiskers. In several animals, a circling motion towards the inoculated side occurred; a syndrome similar to circling disease in sheep with listeriosis (25, 39, 40). These symptoms would progress to generalized paresis and respiratory failure within the next few hours. The animals were killed at the onset of circling motion.

### Bacterial inoculation into triceps surae muscle and sciatic nerve (groups C, D and E)

Seven to eleven days after unilateral injection of bacteria into leg muscle or unilateral exposure of the proximal end of cut sciatic nerve to bacteria, animals developed lumbar paraplegia with bladder paresis and flaccid

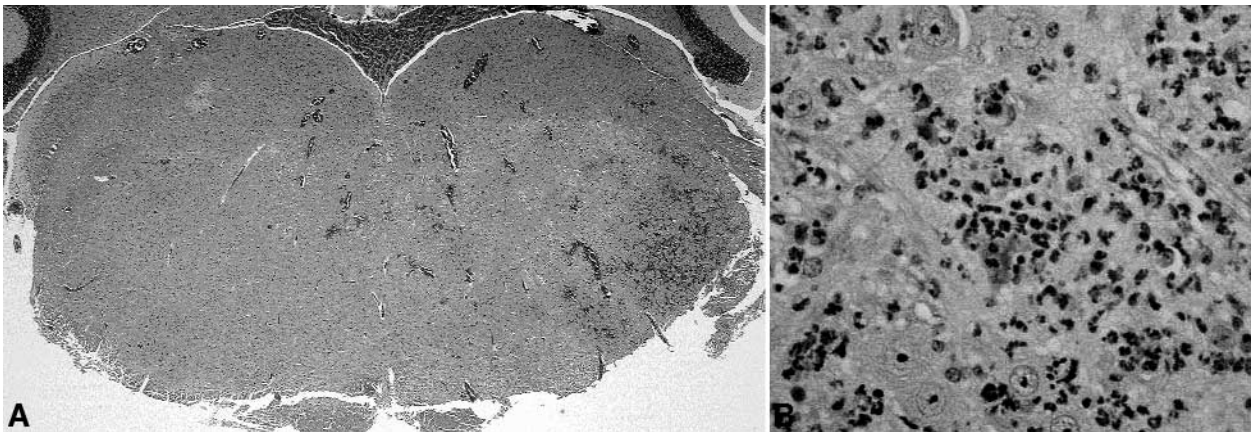


**Figure 1. A**. Murine rhombencephalitis with inflammatory infiltrates in the nucleus of the left cranial nerve V, seven days after inoculation of *L. monocytogenes* into the left whisker area (×2). Unilateral paralysis of whiskers occurred six days after inoculation. **B**. Magnification of the inflammatory infiltrate in figure A (×40).

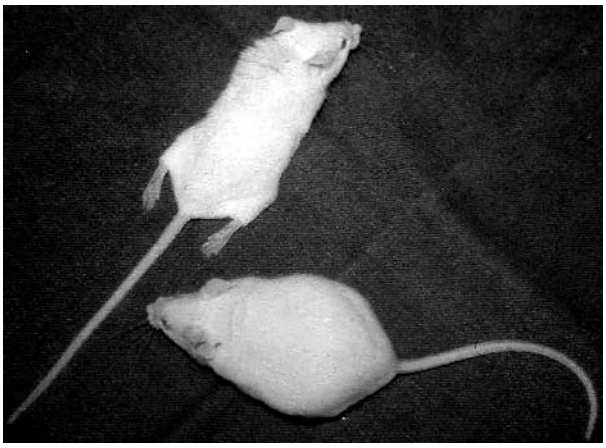

**Figure 2.** Signs of transverse myelitis (bilateral hindleg paresis and flaccid tail) occurring eight days after unilateral injection of *L. monocytogenes* into the left triceps surae muscle (affected animal above, healthy animal below).

paresis of both hind limbs and tail (Figure 2). Interruption by section and removal of 1-2 mm of the sciatic nerve at the mid-thigh level prior to bacterial inoculation in triceps surae muscle prevented development of paraplegia in all cases (n = 5; group E).

### Microscopical findings

In cases of rhombencephalitis, the inflammatory infiltrates (Figure 1B) were predominantly localized in the nuclei of cranial nerves V and VII (Figure 1A). As the infection progressed, bilateral leukocyte infiltration was common. Meningitis occurred in some animals. In cases of myelitis, inflammation was observed within the sciatic nerve at all levels from the inoculation site to the corresponding nerve roots and lumbar segments of the spinal cord (Figure 3 A-D, F). Muscle and connective tissue surrounding the nerve were generally free of inflammation (Figure 3F). The inflammatory infiltrates were subacute with polymorphonuclear and mononuclear leukocytes and occasional microabscesses (Figure 3B, D). Bacteria were present in mononuclear cells and axons in the affected peripheral nerve fascicles and nerve roots. In some nerve roots, the majority of the bacteria were localized within axons (Figure 4 A, B). Little inflammation was present in these cases. Neither inflammation nor bacteria were observed in nerves or nerve roots contralateral to the inoculation site (Figure 3 A, C, E). Interruption of the sciatic nerve at the mid-thigh level prior to bacterial application prevented development of inflammation along the nerve above the site of nerve interruption (group E). Transverse myelitis did not occur in these animals.

### Discussion

The hypothesis suggesting that *L. monocytogenes* may reach the brain by ascending infection along cranial nerves was proposed by Asahi *et al.* in 1957 (3). The same year, Eck described *L. monocytogenes* rhombencephalitis in humans (13). Charlton and Garcia studied spontaneous *L. monocytogenes* rhombencephalitis in sheep and demonstrated unilateral neuritis with intraaxonal bacteria in facial and trigeminal nerves (7, 8). Otter and Blakemore injected *L. monocytogenes* into murine sciatic nerve. The animals developed lumbar myelitis, and bacteria were observed proximally to the inoculation site within sciatic nerve axons (27). Our observations extend these results. Clinical and histopathological signs of left brain stem encephalitis developed after inoculation of bacteria into the left whisker region or the

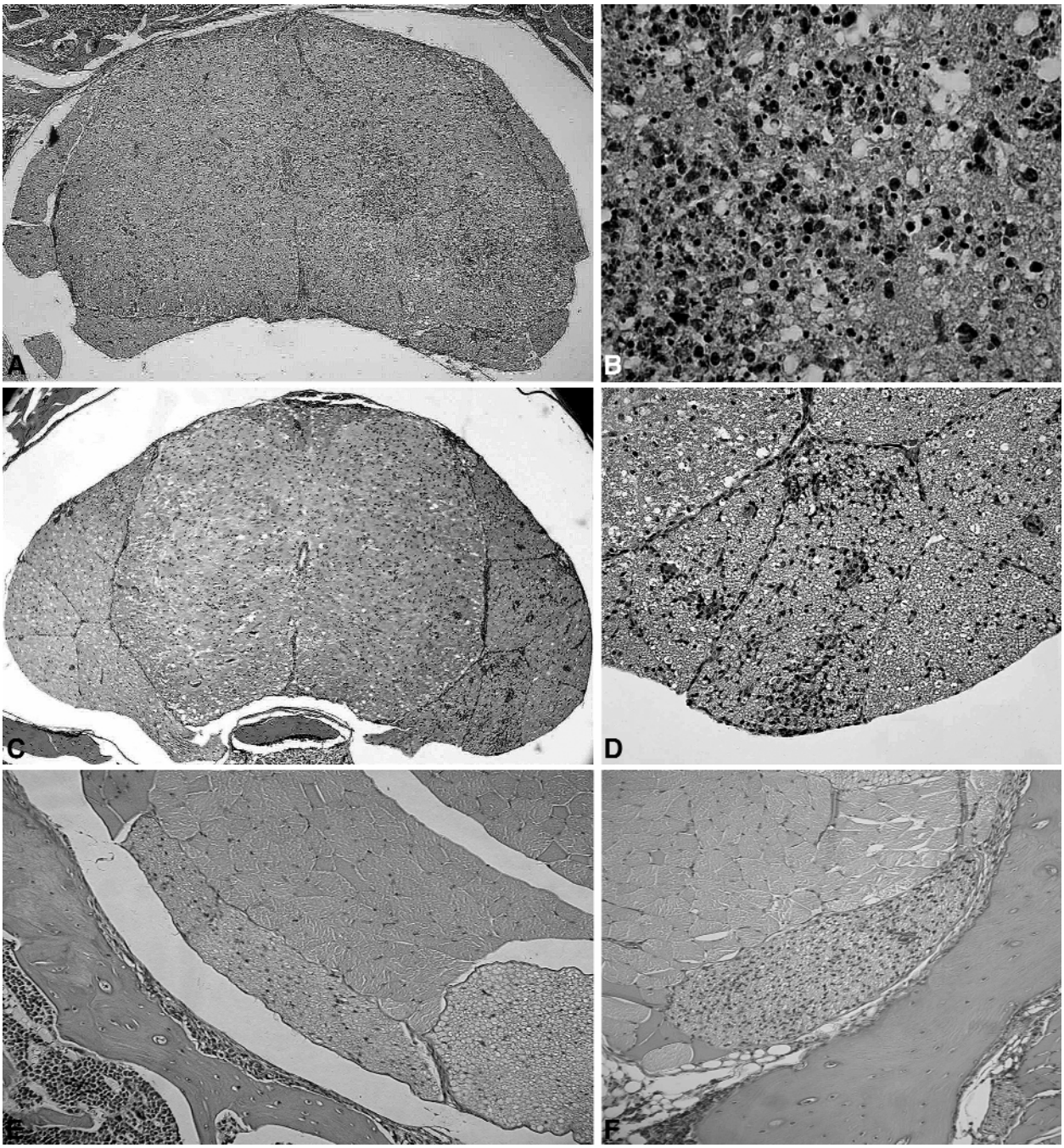


**Figure 3.** Myelitis and unilateral inflammation along sciatic nerve and sciatic nerve roots in animal with signs of transverse myelitis occurring ten days after unilateral injection of *L. monocytogenes* into left triceps surae muscle (all H & E-stained). **A**. Transverse myelitis in the lower lumbar medulla (×4). Note the more severe inflammation on the left side. **B**. Magnification of the inflammatory infiltrates in the left ventral horn in figure A (×40). **C**. Inflammatory infiltrates in ipsilateral (left) ventral nerve roots (×4). **D**. Magnification of the affected ventral nerve root in figure C (×10). **E**. Unaffected right sciatic nerve in the pelvic region (×10). **F**. Inflammatory infiltrates in the left sciatic nerve at the same level as in figure E (×10).

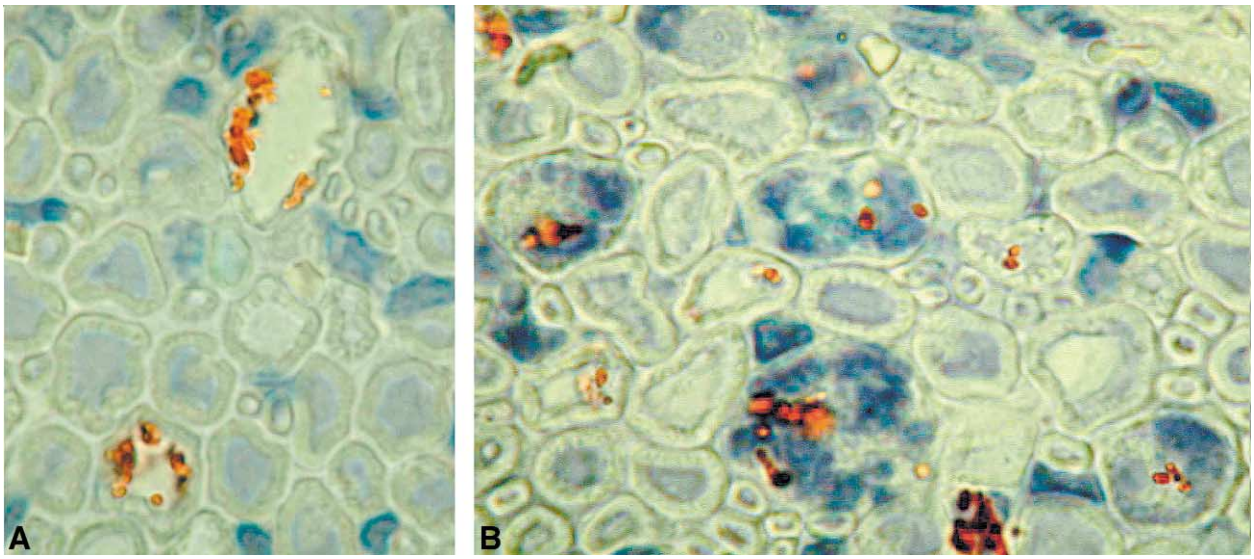


**Figure 4. A** and **B**.Intraaxonal *L. monocytogenes* from nerve root of lower lumbar medulla. Immunostaining with antiserum to *L. monocytogenes* serotype 4 (×100, oil).

proximal end of left cut facial nerve. Lumbar myelitis developed after inoculation of bacteria into the left sciatic nerve or the left hindleg muscle. This did not occur after nerve transsection proximally to the inoculation site. In diseased animals, subacute inflammation with bacteria was present along the neural pathway from the peripheral application site to the corresponding nerve nuclei. In affected nerve roots, bacteria were often detected within axons. Signs of inflammation or intraaxonal bacteria did not occur in contralateral nerves or nerve roots in any animal. Signs of bilateral rhombencephalitis or transverse myelitis invariably occurred in later stages of disease. Otherwise, signs of rhombencephalitis were not observed after inoculation into the leg muscle or the sciatic nerve although fatal septicemia with numerous microabscesses in liver and other organs occurred in some of these animals. Thus, bacterial dissemination into the circulation does not seem to be linked to the development of rhombencephalitis.

In contrast to our evidence above, several experimental studies have concluded that *L. monocytogenes* meningoencephalitis arises by hematogenous bacterial spread. Brain infection after intravenous (4) or subcutaneous (31) inoculation of *L. monocytogenes* gave rise to the suggestion that the organism itself or infected macrophages cross the blood brain barrier. Hematogenous spread was also suggested in a study of experimental listeriosis in gerbils; in this species, rhombencephalitis occurred after bacterial inoculation in the middle ear (5). Meningeal inflammation and perivascular infiltrates occurred in animals in our study, but we have interpreted these as secondary to the infection of the brain parenchyma rather than a primary pathology.

We do not know how *L. monocytogenes* entered axons in our experiments. *L. monocytogenes* predominantly enters non-neuronal cells in mixed cell cultures from brain tissue (12, 29, 30). However, bacterial uptake into neurons readily occurs when mixed cell cultures are exposed to macrophages containing bacteria (12). *L. monocytogenes* may therefore have entered neurons from infected macrophages in these experiments. In intact animals, peripheral nerves are protected by a blood-nerve barrier (1). As the nerve was transected, this barrier was broken in some of our experiments. The blood-nerve barrier may also have been broken due to local inflammation after intramuscular inoculation of bacteria. Bacteria, or macrophages containing bacteria, may therefore have had access to axons in all of our experiments.

Culture studies have demonstrated that phagocytosis of *L. monocytogenes* is induced by the binding of the bacterial surface molecule internalin (23, 28) to E-cadherin on the surface membrane of epithelial cells (26). E-cadherin is present on Schwann cells (14), and may also be present on subtypes of neurons (34). Selective infection of components of the peripheral nervous system may be an important step in the pathogenesis of rhombencephalitis.

In our experiments, both dorsal and ventral roots of animals with transverse myelitis contained intraaxonal bacteria. Ipsilateral inflammation also occurred in the spinal ganglia, and in the ventral and dorsal horns of the

spinal cord. This suggests that *Listeria monocytogenes* may be transported in the axons of both sensory and motor neurons. However, the experiments were not designed to answer whether the bacterium shows tropism for subclasses of neurons.

The mechanism for intraaxonal spread of *L. monocytogenes* is not known. Like mitochondria (18) and several other viruses (20, 22), *L. monocytogenes* may undergo centripetal axonal transport along microtubules. In non-neural cell culture, *L. monocytogenes* (36, 37) and some other intracellular microorganisms (10, 11, 17) form actin tails. The organism thereby obtains motility (19, 35).

The intracellular motility of *L. monocytogenes* is dependent on ActA, a bacterial wall protein which causes polymerization of actin to tail-like structures (6, 21). This process is associated with intracellular movement of bacteria at speeds of 5 μm/s, as studied in infected cell cultures (9, 33). We propose that actin tail formation may serve two functions in the neuroinvasive process: *1)* As observed in vitro, actin dependent motility may facilitate axonal uptake of *L. monocytogenes* from infected non-neuronal cells (11, 32), and *2)* the actin dependent motility may enable *L. monocytogenes* to ascend the whole length of an axonal cylinder. To demonstrate actin tails on intraaxonal bacteria, other techniques than the present must be employed. In this context, it is interesting that Forscher *et al.* have observed organelle motility associated with the formation of actin tails within growing neuronal processes (16). Axonal movement of *L. monocytogenes* may be an example of a more generalized mechanism for actin based organelle transport within neurites.

In conclusion, we have shown that *L. monocytogenes* caused ipsilateral brain stem infection in mice after unilateral bacterial inoculation into facial muscle or nerve. Similarly, lumbar myelitis followed bacterial inoculation into hindleg muscle or sciatic nerve. Intraaxonal bacteria were observed in nerve roots at the side of bacterial inoculation. Nerve section proximal to the inoculation site prevented the spread of the infection to the central nervous system. Together, these observations strongly suggest that *L. monocytogenes* reaches the central nervous system by way of axonal transport. We propose that actin based motility is involved.

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